Chenodeoxycholic acid significantly impacts the expression of miRNAs and genes involved in lipid, bile acid and drug metabolism in human hepatocytes

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Abstract: AIMS: Bile acids (BAs) are important gut signaling hormones, influencing lipid, glucose, and energy homeostasis. The exact mechanisms behind these effects are not yet fully understood. Lately, they have come to the fore as putative therapeutics in metabolic diseases, such as e.g. nonalcoholic fatty liver disease (NAFLD). We elucidate to what extent BAs impacts on the mRNAome and microRNAome in hepatocytes to gather novel insights into the mechanisms behind metabolic and toxicologic effects of bile acids. MAIN METHODS: Five batches of primary human hepatocytes were treated with 50 mol/l chenodeoxycholic acid (CDCA) for 24 or 48h. Total RNA was extracted, size fractionated and subjected to Next Generation Sequencing to generate mRNA and miRNA profiles. KEY FINDINGS: Expression of 738 genes and 52 miRNAs were CDCA dependently decreased, whereas 1566 genes and 29 miRNAs were significantly increased in hepatocytes. Distinct gene clusters controlling BA and lipid homeostasis (FGF(R), APO and FABP family members, HMGCS2) and drug metabolism (CYP, UGT and SULT family members) were significantly modulated by CDCA. Importantly, CDCA affected distinct microRNAs, including miR-34a, -505, -885, -1260 and -552 that systematically correlated in expression with gene clusters responsible for bile acid, lipid and drug homeostasis incorporating genes, such as e.g. SLCO1B1, SLC22A7, FGF19, CYP2E1, CYP1A2, APO family members and FOXO3. SIGNIFICANCE: Bile acids significantly modulate metabolic and drug associated gene networks that are connected to distinct shifts in the microRNAome These findings give novel insights on how BA enfold metabolic and system toxic effects.

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ABSTRACT

Aims: Bile acids (BAs) are important gut signaling hormones, influencing lipid, glucose, and energy homeostasis. The exact mechanisms behind these effects are not yet fully understood. Lately, they have come to the fore as putative therapeutics in metabolic diseases, such as e.g. nonalcoholic fatty liver disease (NAFLD). We elucidate to what extent BAs impacts on the mRNAome and microRNAome in hepatocytes to gather novel insights into the mechanisms behind metabolic and toxicologic effects of bile acids.
Main methods: Five batches of primary human hepatocytes were treated with 50 μmol/l chenodeoxycholic acid (CDCA) for 24 or 48 h. Total RNA was extracted, size fractionated and subjected to Next Generation Sequencing to generate mRNA and miRNA profiles.
Key findings: Expression of 738 genes and 52 miRNAs were CDCA dependently decreased, whereas 1566 genes and 29 miRNAs were significantly increased in hepatocytes. Distinct gene clusters controlling BA and lipid homeostasis (FGF(R), APO and FABP family members, HMGCS2) and drug metabolism (CYP, UGT and SULT family members) were significantly modulated by CDCA. Importantly, CDCA affected distinct microRNAs, including miR-34a, -505, -885, -1260 and -552 that systematically correlated in expression with gene clusters responsible for bile acid, lipid and drug homeostasis incorporating genes, such as e.g. *SLCO1B1*, *SLC22A7*, *FGF19*, *CYP2E1*, *CYP1A2*, *APO* family members and *FOXO3*.
Significance: Bile acids significantly modulate metabolic and drug associated gene networks that are connected to distinct shifts in the microRNAome These findings give novel insights on how BA enfold metabolic and system toxic effects.

1. Introduction

Bile acids (BAs) are important endogenous compounds responsible for the efficient absorption of lipid-soluble compounds in the intestine. Semiquantitative BA derivatives, such as e.g. obeticholic acid (OCA), are currently discussed as future treatment option for different metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD), the most common liver disease in the western world [1]. There is an urgent need to better understand how BAs enfold their effects on metabolic pathways and on their own homeostasis to better estimate safety and efficacy of these compounds.

The effects of BAs on metabolism are triggered by their interaction with the nuclear receptor FXR, which leads to improvement of steatosis and fibrosis in NAFLD [1,2] and a bettering of hepatic insulin sensitivity [3]. By interacting with FXR, BAs also regulate their own homeostasis via negative and positive feedback loops [4], thus preventing cells from an

Abbreviations: ABC, ATP-binding cassette transporter; AGPAT2, 1-acylglycerol-3-phosphate *O*-acyltransferase; AhR, arylhydrocarbon receptor; AKR1C1, aldo-keto reductase 1C1; APO, apolipoprotein; ASBT, apical sodium dependent bile acid transporter; BAs, bile acids; BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; CPT1A, carnitine palmitoyltransferase 1A; CYP, cytochrome P450; DM, drug metabolism; DMSO, dimethyl sulfoxide; FABP, fatty acid-binding protein; FASN, fatty acid synthase gene; FDR, false discovery rate; FXR, farnesoid X receptor; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; LDLR, low density lipoprotein receptor; mRNA, messenger RNA; miRNA, microRNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NPC1L1, Niemann-Pick C1-like 1 gene; NTCP, Na^+-taurocholate cotransporting polypeptide; OATP, organic anion transport protein; OST, organic solute transporter; PCR, polymerase chain reaction; PHHs, primary human hepatocytes; PPAR-γ, peroxisome proliferator-activated receptor; RT-PCR, real-time PCR; S1PR, sphingosine-1-phosphate receptor; SHP, small heterodimer partner; SLC, solute carrier family; STARD3, StAR-related lipid transfer domain protein 3; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; UTR, untranslated region.

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Table 1
Clinical characteristics of liver cell donors.

Patient No	Gender	Age	Main diagnosis	drugs
1	Female	Between 71 and 80	Colon carcinoma and liver metastases	None
2	Female	Between 70 and 79	GIST tumor	None
3	Female	Between 51 and 60	Rectosigmoid carcinoma with liver metastases	State after 6 cycles of oxaliplatin/folinic acid(FUFOX) Avastatin
4	Female	Between 51 and 60	Liver metastasis after kidney cancer	Bisoprolole *L*-thyroxine Zopiclone Anagrelide
5	Female	Between 31 and 40	Hepatocellular carcinoma	Metamizole

intracellular BA overload with toxic effects [5]. The enterohepatic circulation and homeostasis of BAs are ensured by a coordinated action of BA uptake and efflux transporters. Bile acid transporters include amongst others the apical sodium-dependent bile acid transporter (ASBT) and organic solute transporters α/β (OSTα/β) in the intestine, and the Na^+-taurocholate co-transporting polypeptide (NTCP), the organic anion-transporting polypeptides OATP1B1 and OATP1B3 and the bile salt export pump (BSEP) in hepatocytes [6].

As ligands for the nuclear receptors FXR, PXR or CAR, BAs regulate the expression of several genes that are important for both BA homeostasis and drug metabolism, such as e.g. OATP1B1, OATP1B3 and CYP3A4. This effect may give space for interactions between BAs and therapeutics and may affect drug exposure margins with consequences for drug safety and efficacy. By interacting with several other receptor molecules, besides FXR, such as muscarinic receptors or G protein-coupled receptors (i.e. TGR5) [7,8], BAs influence lipid and energy homeostasis. TGR5 triggers weight loss upon activation by BAs [9–11] and has a positive impact on glucose tolerance by inducing the secretion of glucagon-like-peptide 1 (GLP-1) from intestinal enteroendocrine cells [12].

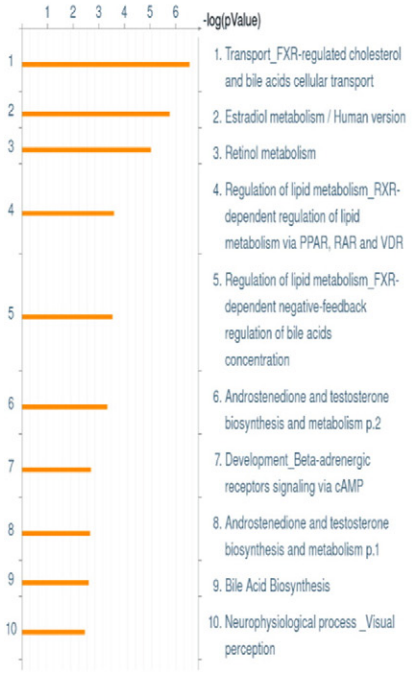
Notwithstanding the role of genetic susceptibility factors in the pathogenesis of obesity and diabetes [13–16], the complex interplay of genetically, epigenetically and microRNA (miRNA)-driven factors is becoming increasingly evident [17–20]. In particular, shifts in the concentrations of miRNAs, small noncoding molecules that inhibit mRNA translation, can lead to rapid changes in protein expression [21]. MiRNAs belong to a relatively small pool of molecules controlling the expression of major parts of the genome. Thus, expression changes of only few miRNAs can broadly impact the functional integrity of different metabolic and signaling pathways.

In the current study we investigate the effect of the BA chenodeoxycholic acid (CDCA) on the expression of the miRNAome and mRNAome in primary human hepatocytes (PHHs) and assess to what extent CDCA induces systematic shifts in gene networks responsible for BA and lipid homeostasis as well as drug metabolism. BA-induced shifts in the miRNA profile are set in context to the investigated networks to elucidate novel miRNA driven regulatory pathways influencing the expression of the mentioned gene networks.

2. Material and methods

2.1. Primary human hepatocytes (PHH)

The study was approved by the Ethics Committee of the Canton of Zurich, Switzerland (study number EK-680) and the Human Tissue and Cell Research (HTCR) Foundation. The HTCR-process that included written informed consent was approved by the Ethics Committee of the Medical Faculty of the Ludwig Maximilians University (approval number 025-12) and complied with the Bavarian Data Protection Act. PHH were obtained from five patients in Germany who were



p-value	FDR	No of objects*	Network Objects
0.000000	0.000075	7/42	SHP, MDR1, OST alpha/beta, MDR3, BSEP, OSTalpha, OSTbeta
0.000002	0.000155	6/35	SULT1E1, CYP3A4, UGT1A10, CYP2C8, CYP1A1, CYP1A2
0.000002	0.000155	6/35	SULT1E1, CYP3A4, UGT1A10, CYP2C8, CYP1A1, CYP1A2
0.000002	0.000163	6/37	SULT1E1, CYP3A4, UGT1A10, CYP2C8, CYP1A1, CYP1A2
0.000007	0.000348	7/66	CYP4A11, CYP3A4, UGT1A8, CYP2C8, CYP2E1, CYP1A1, CYP1A2
0.000010	0.000431	7/70	CYP4A11, CYP3A4, UGT1A8, CYP2C8, CYP2E1, CYP1A1, CYP1A2
0.000289	0.010765	4/30	HMGCS2, SHP, RARalpha, ADHG
0.000329	0.010765	4/31	FGF19, SHP, CYP3A4, CYP7A1
0.000529	0.015403	4/35	UGT1A9, AKR1C1, UGT1A8, UGT1A10
0.000590	0.015467	4/36	UGT1A9, AKR1C1, UGT1A8, UGT1A10

Fig. 1. Gene enrichment analysis showing signaling pathways most affected by CDCA.

undergoing liver resection because of liver metastases in association with primary tumors in colon or kidney or because of hepatocellular carcinoma. The clinical characteristics of the patients are summarized in Table 1 (supplementary information). PHHs were prepared as earlier described [22] and kept in six-well plates in hepatocyte maintenance medium supplemented with UltraGlutamine for approximately 5 h before further treatment procedures. PHHs were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 at atmospheric pressure.

2.2. Cell treatment and whole RNA isolation

PHHs of 5 patients were kept in 6 well plates. 24 h later duplicate wells of each cell batch were treated with chenodeoxycholic acid (CDCA) or dimethyl sulfoxide (DMSO) (vehicle control) (both from Sigma-(both from Sigma-Aldrich, Buchs, Switzerland). One CDCA and one DMSO treated well of each cell batch were harvested together 24 h or 48 h after cell treatment using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for combined DNA and RNA isolation. Subsequently, total RNA was purified using the miRNeasy kit from Qiagen (QIAGEN, Hombrechtikon, Switzerland).

2.3. Next generation sequencing

For library preparation the quality of the isolated RNA was evaluated using a Qubit® (1.0) Fluorometer (Life Technologies, Carlsbad, CA, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only those samples with a 260 nm/280 nm ratio between 1.8 and 2.1 and a 28S/18S ratio of 1.5–2 were further processed. 3′ and 5′ RNA adapters were ligated to total RNA samples (1 μg) using the TruSeq small RNA Sample Prep Kit v2 (Illumina, Inc., San Diego, CA, USA). Ligated samples were reverse-transcribed into double-stranded cDNA and fragments containing TruSeq adapters on both ends were selectively enriched by polymerase chain reaction (PCR). The small RNA fraction (145–160 bp) was selected and isolated by polyacrylamide gel electrophoresis. The quality and quantity of the enriched libraries were validated using a Qubit® (1.0) Fluorometer and the Caliper GX LabChip® GX (Caliper Life Sciences, Inc., Hopkinton, MA, USA). The libraries were diluted to 10 nM in Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20. For cluster generation and sequencing the TruSeq PE Cluster Kit v3-cBot-HS was used with 10 pM of pooled normalized libraries on the cBOT system (Illumina, Inc.). Sequencing was performed on the Illumina HiSeq 2000 using the TruSeq SBS Kit v3-HS (Illumina, Inc.).

Table 2
Effect of CDCA on the expression of genes involved in bile acid homeostasis and drug metabolism in primary human hepatocytes after 48 h.

Gene name*	log_2 ratio	Fold change	*p*-Value	FDR
Up-regulated genes				
Bile acid homeostasis				
FGF19	5.078	33.78	3.541xE-07	9.624xE-05
SLC51B	4.934	30.57	1.091xE-15	2.958xE-12
NR0B2	2.85	7.21	9.207xE-11	8.158xE-08
ABCB11	2.195	4.58	2.194xE-09	1.177xE-06
SLC51A	2.194	4.58	8.15xE-16	2.768xE-12
ABCB4	1.603	3.04	5.099xE-10	3.149xE-07
ABCG5	0.8736	1.83	0.0015	0.065
*SLCO1B3***	0.5981	1.51	0.0045	0.124
ABCG8	0.4935	1.41	0.0438	0.434
Drug metabolism				
UGT2B10	1.264	2.4	0.0006	0.033
SULT1C2	1.13	2.19	0.0393	0.412
UGT1A8	0.9499	1.93	0.021	0.298
UGT2B4	0.7462	1.68	0.0098	0.196
UGT1A3	0.5976	1.51	0.0269	0.34
UGT1A1	0.5671	1.48	0.0416	0.425
PPARG	0.5001	1.41	0.0431	0.432
Down-regulated genes				
Bile acid homeostasis				
*EPHX1***	−0.4618	0.73	0.035	0.389
NR1I3	−0.5675	0.67	0.0106	0.207
SLCO1B1	−0.5869	0.67	0.0046	0.126
BAAT	−0.8562	0.55	0.0035	0.107
*CYP3A4***	−1.734	0.3	2.055xE-13	3.49xE-10
CYP7A1	−6.271	0.01	9.197xE-18	4.686xE-14
Drug metabolism				
AHR	−0.4569	0.73	0.0413	0.423
UGT1A6	−0.5588	0.68	0.0127	0.229
NR1I3	−0.5675	0.67	0.0106	0.207
SLCO1B1	−0.5869	0.67	0.0046	0.126
UGT2A3	−0.7303	0.6	0.0026	0.09
SULT1B1	−0.7455	0.6	0.0017	0.067
UGT1A7	−0.9585	0.51	0.0423	0.429
SLC22A7	−1.054	0.48	0.0189	0.282
CYP2C8	−1.605	0.33	8.14E-07	<0.001
SULT1E1	−1.679	0.31	0.0014	0.063
SLC22A1	−1.726	0.3	8.55E-12	1.09E-08
CYP1A2	−1.921	0.26	1.05E-07	3.23E-05
CYP1A1	−1.954	0.26	1.96E-05	0.002
UGT1A9	−2.095	0.23	6.84E-05	0.007
CYP2E1	−2.556	0.17	2.03E-21	2.07E-17

* Genes involved in drug metabolism or bile acid homeostasis with a FDR < 0. 46 a *p*-value < 0.05.
** Involved in both bile acid homeostasis and drug metabolism or drug transport.

Table 3
Effect of CDCA on the expression of genes involved in lipid homeostasis in primary human hepatocytes after 48 h.

Gene name*	log_2 ratio	Fold change	*p*-Value	FDR
Bile acid dependently up-regulated genes				
FABP3	2.981	7.9	0.0001	0.009
APOL3	1.464	2.76	1.50E-10	1.09E-07
FGFR2	1.42	2.68	8.02E-06	0.001
LDLR	1.339	2.53	4.20E-13	6.58E-10
PPARD	1.256	2.39	2.18E-06	<0.001
FGF21	1.25	2.38	0.0281	0.347
FASN	1.185	2.27	0.0081	0.174
PCSK9	1.182	2.27	3.95E-06	0.001
S1PR2	1.146	2.21	0.0122	0.224
S1PR1	0.9341	1.91	2.49E-05	0.003
PRKCE	0.8938	1.86	0.0019	0.073
APOA2	0.8007	1.74	0.003	0.098
SCARB1	0.691	1.61	0.0017	0.068
CPT1A	0.6706	1.59	0.0018	0.071
SREBF2	0.6289	1.55	0.0071	0.162
LDLRAP1	0.6005	1.52	0.0061	0.149
NPC1	0.5843	1.5	0.0024	0.085
NPC1L1	0.5734	1.49	0.0014	0.061
SLC27A2	0.5539	1.47	0.0034	0.106
STARD3	0.5293	1.44	0.02643	0.336
FGF2	0.526	1.44	0.02203	0.305
PRKCA	0.5093	1.42	0.0053	0.137
FOXO3	0.5087	1.42	0.0284	0.349
AGPAT2	0.5037	1.42	0.0187	0.281
CREBBP	0.45	1.37	0.0355	0.391
FGFRL1	0.4191	1.34	0.0493	0.459
ATF6B	0.416	1.33	0.0352	0.389
Bile acid dependently down-regulated genes				
ACAT1	−0.432	0.74	0.02	0.291
PEX3	−0.4953	0.71	0.0115	0.216
AKR7A3	−0.5941	0.66	0.0041	0.118
APOM	−0.6031	0.66	0.0064	0.151
ACADSB	−0.6303	0.65	0.0008	0.042
APOC4	−0.7548	0.59	0.0086	0.182
AKR1C1	−0.8871	0.54	0.0161	0.261
APOH	−0.9548	0.52	0.0004	0.027
ANGPTL3	−0.9995	0.5	0.0009	0.045
ACAD11	−0.9997	0.5	2.95E-07	8.36E-05
APOA4	−1.059	0.48	0.0135	0.239
AKR1C4	−1.069	0.48	7.84E-08	2.66E-05
FABP7	−1.964	0.26	0.0051	0.134
HMGCS2	−2.128	0.23	0.0001	0.01

* Genes involved in lipid homeostasis (FDR < 0. 46 and *p*-value < 0.05).

2.4. Processing of NGS data

RNA sequence reads were quality-checked using the software package fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) which computes various quality metrics for the raw reads. MessengerRNA sequencing reads were aligned to the genome and transcriptome using the R statistics package tophat v. 1.3.3 with default options. Before mapping, the low quality ends of the reads were clipped. The fragment length parameter was set to 100 bases with a standard deviation of 100 bases. Based on these alignments the distribution of the reads across genomic features was assessed. Isoform expression was quantified using the RSEM algorithm (R statistics package rsem) [23]. MicroRNA sequencing reads were aligned to the genome and quantified using ncPro-seq (http://www.ncbi.nlm.nih.gov/pubmed/23044543).

2.5. TaqMan analysis

Messenger RNA quantification was performed by transcribing 1.5 μg mRNA into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's recommendations. cDNA samples were diluted 1:5 and subsequently used in real-time PCR analyses (RT-PCR) by mixing 2 μl of cDNA and 8 μl of RT-PCR Universal Fast Master Mix specific to the respective cDNA target (TaqMan® Gene Expression Assays, Life-Technologies). β-actin was used to normalize measurements. miRNAs were quantified by transcribing 10 ng of extracted RNA into cDNA (TaqMan® miRNA Reverse Transcription Kit, Applied Biosystems, Rotkreuz, Switzerland) using stem-loop reverse transcription primers specific for the respective miRNA molecule (TaqMan® MicroRNA Assays, Life Technologies). RT-PCR analyses were performed using 0.67 μl of cDNA and 9.3 μl of the target-specific RT-PCR Universal Fast Master Mix (Applied Biosystems). All measurements were performed in triplicate.

2.6. Statistics

Generalized Linear Models (GLM) were used for statistical analysis comparing the mRNA and miRNA expression values across the different treatment conditions after NGS sequencing. One-sample t-tests were performed to compare the effects of CDCA on the expression of miRNAs versus DMSO as measured in TaqMan analysis. MiRNAs and mRNAs were correlated by Pearson's correlation analyses. Correlation diagrams were obtained using the "corrplot" library of the R-project, of mRNA and miRNA expression levels in both CDCA and DMSO treated cell lines, respectively. The statistical packages SPSS 22 and Graphpad Prism version 5 were used for statistical analyses. p-Values < 0.05 were considered significant.

3. Results

3.1. Effect of CDCA on genes involved in bile acid synthesis and transport, lipid, retinol and estradiol metabolism in hepatocytes

PHHs were treated with CDCA or DMSO (empty vehicle) for either 24 or 48 h to study the effect on the mRNA and miRNA expression profile. Changes in the expression of mRNAs were in general more pronounced after 48 h, and these results are therefore specifically discussed in the following section. Expression of 738 genes was significantly decreased and expression of 1566 genes was significantly increased (False Discovery Rate (FDR) in both cases ≤46%) after 48 h of CDCA treatment. When considering a FDR up to 30%, expression of 432 genes was significantly increased and expression of 1011 genes was significantly decreased in a BA-dependent manner. Gene enrichment analysis using the bioinformatics tool Metacore™ (Thomson Reuters) revealed that genes with altered expression in response to CDCA belong primarily to gene networks involved in BA and lipid transport and metabolism, as well as estradiol and retinol metabolism (Fig. 1). As expected, CDCA increased expression of FXR-inducible genes such as *ABCB11* (BSEP), *FGF19*, *NR0B2* (SHP) or *SLC51A* and *SLC51B* (OSTα, OSTβ) (Table 2).

Genes involved in the regulation of lipid homeostasis that showed altered expression in response to CDCA included several members of the apolipoprotein (*APO*) and fatty acid- binding protein (*FABP*) family, *SLC27A2* and *STARD3*, as well as genes involved in cholesterol, lipid and fatty acid (FA) synthesis and metabolism, such as *CPT1A*, *HMGCS2*, *FASN*, *ACAT1* and *PCSK9* (Table 3). Within signaling pathways influencing lipid homeostasis, *LDLR*, *PRKCA1*, *PPARδ*, *AGPAT2* and *SREBF2* were affected by CDCA. However, nuclear receptors known to control both BA and lipid homeostasis, e.g., FXR and LXR, were not significantly changed in their expression.

3.2. Effect of CDCA on the expression of drug-metabolizing enzymes

As shown in Table 2, CDCA modulated the expression of many genes involved in drug transport and phase I and II drug metabolism. Amongst drug-metabolizing cytochrome P450 enzymes, the expression of *CYP2E1*, *CYP1A1*, *CYP1A2*, *CYP2C8*, and *CYP3A4* was significantly decreased by CDCA. Amongst phase II enzymes, members of the UDP-glucuronosyl-transferase (UGT) and sulfotransferase (SULT) families in particular showed altered expression in response to CDCA. Specifically, expression of *UGT1A6*, *1A7* and *1A9*, *SULT1E1* and *SULT1B1* was decreased and expression of *UGT1A1*, *2A8* and *2B10*, and *SULT1C2* was increased by CDCA. Transcription factors that regulate drug metabolism and excretion, such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) or peroxisome proliferator-activated receptor γ (PPARγ) also showed altered expression in response to CDCA (Table 2).

Table 4
Effect of CDCA on the expression of miRNAs in primary human hepatocytes after 48 h.

miRNA*	$\log_2$ ratio	Fold change	p-Value	FDR
Bile acid dependently up-regulated miRNAs				
hsa-mir-552	1.588	3.006	0.0073	0.126
hsa-mir-1260a	1.243	2.367	<0.0001	0.002
hsa-mir-149	1.207	2.309	0.0052	0.115
hsa-mir-1260b	1.186	2.275	<0.0001	0.004
hsa-mir-3651	1.129	2.187	0.0032	0.115
hsa-mir-4485	0.9179	1.889	0.0038	0.115
hsa-mir-3607	0.8542	1.808	0.0043	0.115
hsa-mir-505	0.8398	1.79	0.0035	0.115
hsa-mir-6723	0.7282	1.657	0.0052	0.115
hsa-mir-15b	0.7139	1.64	0.0036	0.115
hsa-mir-328	0.7	1.625	0.0081	0.133
hsa-mir-92a-2	0.6913	1.615	0.0091	0.133
hsa-mir-204	0.5362	1.45	0.0092	0.133
hsa-mir-885	0.4937	1.408	0.0097	0.133
Bile acid dependently down-regulated miRNAs				
hsa-mir-34a	−0.44	0.737	0.0087	0.133
hsa-mir-30a	−0.5096	0.702	0.0055	0.116
hsa-mir-98	−0.6309	0.646	0.0062	0.122
hsa-mir-5590	−0.8854	0.541	0.0036	0.115
hsa-mir-2355	−0.9401	0.521	<0.0001	0.004
hsa-mir-190a	−1.1	0.467	0.0022	0.115
hsa-mir-190b	−1.266	0.416	0.0096	0.133
hsa-mir-2467	−1.421	0.373	0.0038	0.115
hsa-mir-2114	−1.653	0.318	0.005	0.115
hsa-mir-452	−1.847	0.278	<0.0001	0.002
hsa-mir-486	−2.285	0.205	0.0047	0.115
hsa-mir-486-2	−2.287	0.205	0.0048	0.115
hsa-mir-451b	−2.461	0.182	0.0069	0.125
hsa-mir-451a	−2.461	0.182	0.0069	0.125
hsa-mir-6503	−2.9	0.134	0.0009	0.073

* Shown are all CDCA dependently modulated microRNAs with a FDR ≤ 0.133 and a p-value < 0.01.

3.3. The impact of CDCA on the miRNA expression profile in primary human hepatocytes

Whereas only few changes in the miRNA profile were observed after 24 h' treatment of PHHs with CDCA, expression of 52 miRNA molecules was significantly increased and that of 29 miRNAs was significantly decreased after CDCA treatment for 48 h, as shown in Table 4 (FDR < 0.133, $p < 0.01$). Results were confirmed by TaqMan analysis remeasuring and confirming the CDCA dependent downregulation of 3 microRNAs (miR-6503, miR-486 and miR-223) in three separate hepatocyte batches. These microRNAs belong to the group of miRNA molecules most strongly affected by CDCA, thus, allowing a confirmatory trend determination in expression by TaqMan analyses. Other microRNAs affected by CDCA included miR-552, miR-149, and miR-886 (increased) and miR-34a, miR-30a, miR-452, miR-486 and miR 190a and 190b (decreased). Figs. 2 and 3 illustrate the CDCA-dependent miRNA profile as a volcano plot and heatmap, respectively. The heatmap integrated cluster analysis shows considerable interindividual variability in the miRNA expression profile following CDCA treatment.

3.4. Correlation of CDCA-induced changes in miRNA levels with mRNA expression

To evaluate the extent to which miRNAs modulated by CDCA could influence the expression of genes involved in BA synthesis, transport and metabolism, Pearson's correlation analyses were performed. Only those miRNAs (shown in Table 4) that were predicted to bind to at least three mRNAs contained in the gene cluster regulating BA homeostasis (Table 2), lipid metabolism (Table 3) and drug metabolism (Table 2) were included in the correlation analyses. Binding of miRNAs was predicted using the bioinformatics tool mirDIP (http://ophid.utoronto.ca/mirDIP) [24]. As shown in Fig. 4 a distinct cluster of mRNAs consisting of *ABCG5* and *ABCG8*, *SLC22A7*, *NR0B2*, *SLC51B*, *SLC10A1*, *CYP3A4* and *FGF19* is inversely associated with the expression of miR-34a upon CDCA treatment. The same gene cluster appears to be strongly positively correlated with miRNAs −885, −15b and −505, leading to the hypothesis that miRNA and mRNA clusters may be regulated by common transcriptional pathways. As demonstrated in Fig. 5, especially microRNA-1260a shows strong inverse correlations with several important genes involved in lipid homeostasis, including *AGPAT2*, *S1PR2*, *CPT1A*, *APOM*, *AKR1C1* and *LDLR* upon CDCA treatment pointing to an inhibitory effect of miR-1260a on the expression of this gene battery. In contrast to miR-1260a, miRNA-98 that is strongly inversely correlated with a gene battery including amongst others *NPC1L1*, *HMGCS2*, *AGPAT2*, *FASN*, *STARD3* and *S1PR2l*, loses many of those inverse associations upon CDCA treatment. This observation points to a less strong regulatory impact of miR-98 on the lipid gene network upon higher hepatic BA concentrations. Fig. 6 demonstrates the correlation behavior of mRNAs belonging to the drug metabolism network and distinct microRNAs. Interestingly miRNA-1260 comes here to the fore again, now with two family members -1260a and -1260b, showing both strong positive correlations with the drug metabolizing enzymes *SULT1E1*, *CYP1A1* and *CYP1A2* and *CYP2E1*. >Less strong but still relevant positive associations in gene expression are also observed with the UGT family members *UGT2A3*, *UGT1A7*, *UGT1A8* and *CYP2C8*. This observation suggests a connection between miR-1260 expression and the

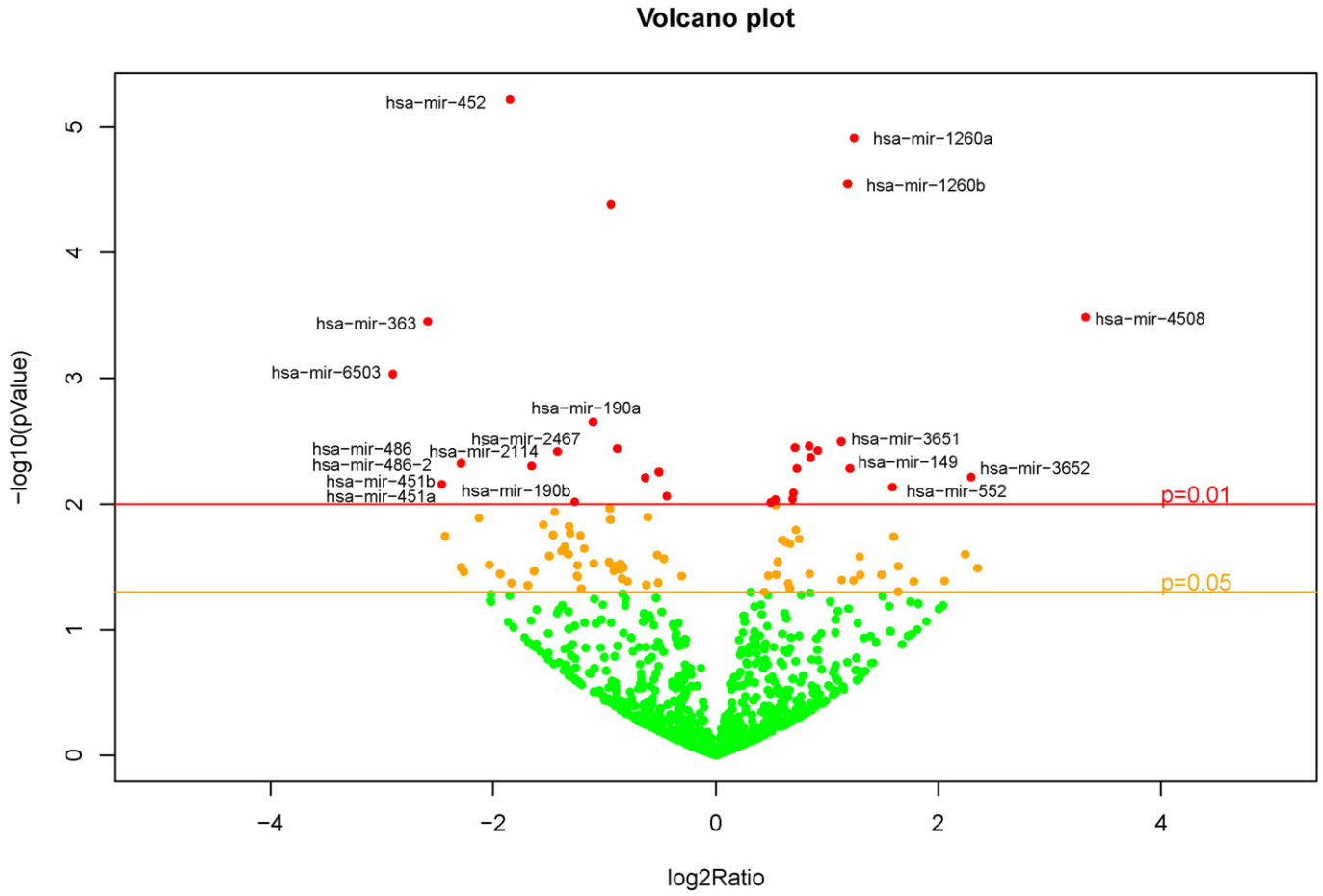


Fig. 2. Effect of CDCA on the global miRNA profile in five batches of primary human hepatocytes. Dots represent the average fold-change of miRNAs shown as $\log_2$ values (x-axis) and corresponding p-values represented as $-\log_{10}$ values (y-axis) when comparing CDCA and empty vehicle (DMSO) treated cells. Red: miRNAs with p-values <0.01. Yellow: miRNAs with p-values <0.05. Green: miRNAs with p-values >0.05. Labeled miRNAs represent molecules that showed a ≥ 2fold increase or decrease.

expression of the mentioned genes, either through a common regulatory element of via indirect signaling pathways involving miRNA-1260 as regulatory compound with impact on e.g. an inhibitory transcription factor regulating the mentioned gene battery.

4. Discussion

We systematically investigated the effect of CDCA on the mRNAome and miRNAome in human hepatocytes. Using CDCA as a model substance we demonstrate that BAs have the ability to profoundly change the transcriptional pattern of gene networks involved in BA and lipid homeostasis, as well as drug metabolism and disposition. These results together with our observation that distinct microRNAs appear to be bile acid dependently changed in expression provide important novel insights into the regulatory mechanisms behind systematic effects of BAs that are of both liver therapeutic and toxicological relevance.

CDCA had both enhancing and suppressing effects on the expression of miRNAs, with the majority of the miRNA molecules having decreased levels under CDCA treatment. Importantly, we show that CDCA modulates the expression of distinct miRNAs that appear to be connected to the expression of gene clusters within the bile acid, lipid and drug homeostasis associated gene networks. MicroRNA-34a is CDCA dependently and strongly inversely correlated with key genes involved in BA homeostasis including FGF19, NR0B2 (SHP), OSTα/β, ABCG5/ABCG8, and SLC22A7. This finding suggests a key role of miR-34a in the autoregulation of bile acid homeostasis. We observed a strong downregulation of miR-34a, which is well in line with results published by Castro et al. (2013), showing a suppressive effect of ursodeoxycholic acid in patients suffering from NAFLD [25]. The suppressive effect of CDCA on miR-34a expression suggests also consequences for NAFLD development, and the regulation of energy homeostasis and cancer related pathways. MicroRNA-34a has been shown to SIRT-dependently regulate brown fat formation. The observed upregulation of serum miR-34a in NAFLD patients underlines the importance of miR-34a in metabolic diseases [26]. MiRNA-34a has been studied in the context of cancer development and tumor growth. Associations with disease development, prognosis and severity have been described for bladder, breast, liver and colon cancer as well as lymphoma [27–30]. Besides miR-34a, we detected a distinct microRNA cluster, composed of miR-885, miR-505 and miR-15b, to be CDCA dependently modulated in expression. These microRNAs were significantly upregulated and showed, in contrast to miR-34a - a positive correlation with the group of genes inversely associated with the expression of miR-34a. It is conceivable that these three miRNAs and the associated BA gene cluster are co-regulated by a common transcriptional pathway that is activated by CDCA. MiRNA-885 has been shown to play a role in the pathogenesis of different cancer types [31,32] and has also been discussed as a potential serum marker for the detection of ongoing liver pathologies [33]. MiR-15b has been shown to be up-

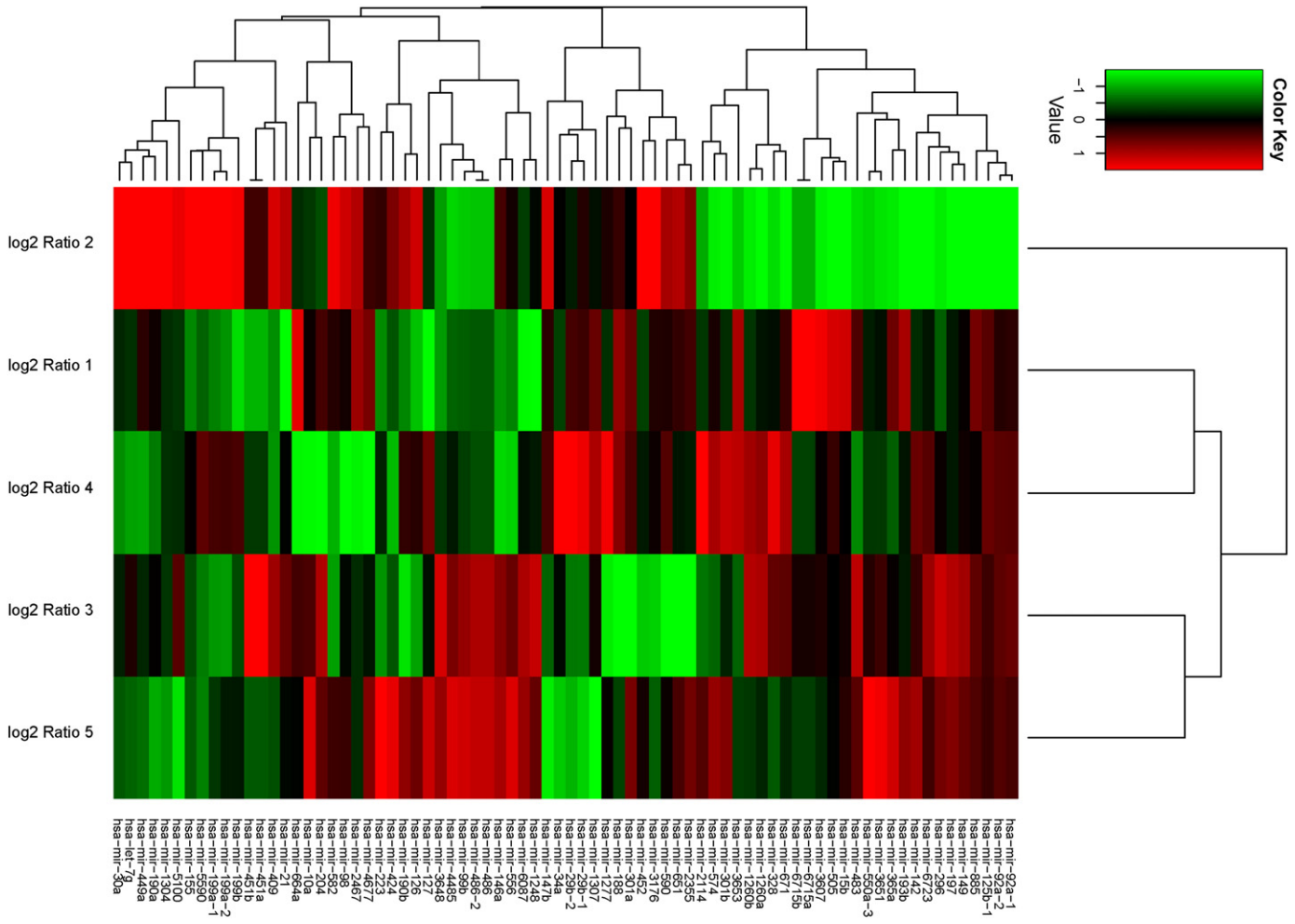


Fig. 3. Heat map showing CDCA-induced changes in miRNA profile clustering in five batches of primary human hepatocytes. MiRNAs with changes of at least 50% and p-values <0.05 are shown. Red: increased miRNAs. Green: decreased miRNAs.

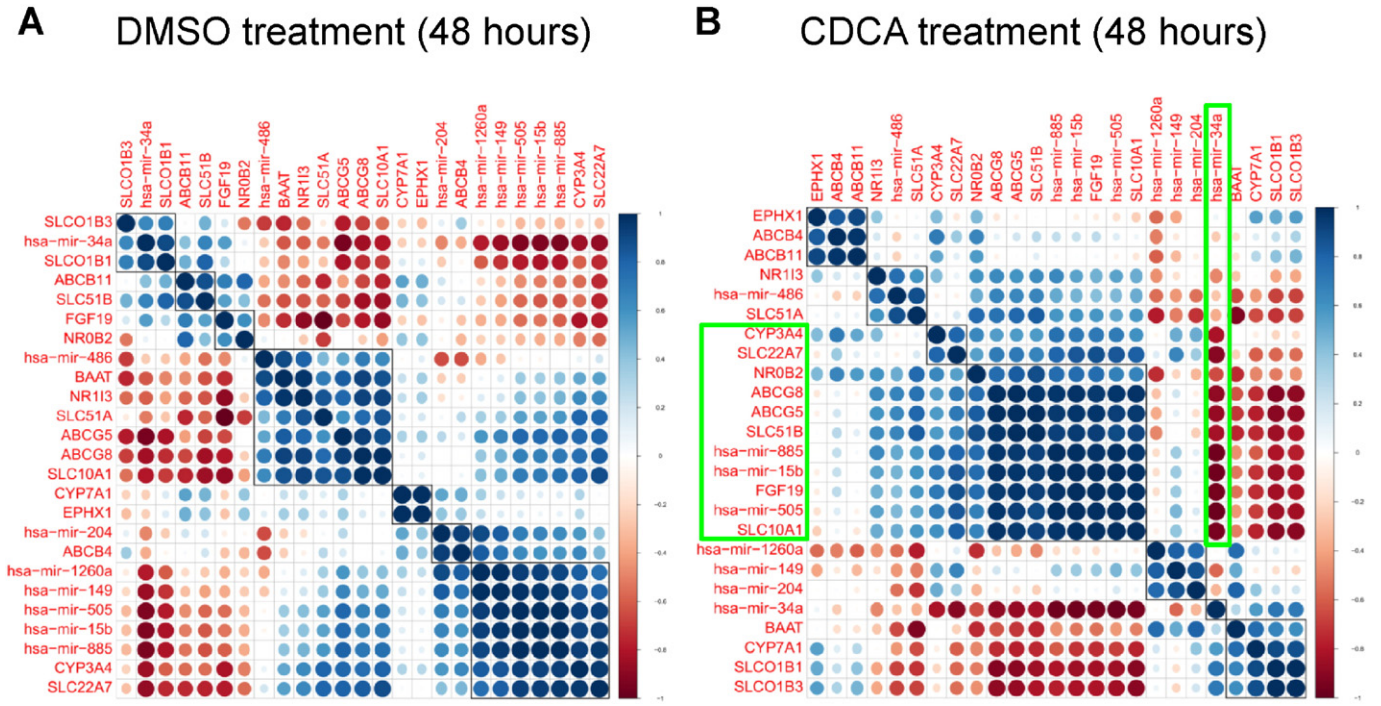


Fig. 4. Pearson's correlation analyses on mRNA and miRNA expression levels taking genes from the BA gene cluster into consideration. DMSO treatment (A), CDCA treatment (B). MicroRNAs with predicted binding sites in at least three target genes within the bile acid (BA) gene cluster and significantly up- analyses on mRNA and miRNA expression levels taking genes from the BA gene cluster into consideration. DMSO treatment (A), CDCA treatment (B). MicroRNAs with predicted binding sites in at least three target genes within the bile acid (BA) gene cluster and significantly up– or downregulated (FDR ≤ 0.133) were included. Shown are BA mRNA targets that were significantly up or downregulated by CDCA (FDR < 0.46). Blue, positive correlation; red, inverse correlation. A distinct gene cluster composed of important BA homeostasis regulating genes, comprising *SLC51A*, *CYP3A4*, *SLC22A7*, *NR0B2*, *ABCG5/8*, *SLC51B*, *FGF19* and *SLC10A1* appears to be strongly inversely correlated with miRNA-34a and positively correlated with the microRNAs miR-885, miR-15b, and miR-505 upon CDCA treatment (highlighted in green).

regulated in NAFLD, and thus, has been suggested to play a role in the pathogenesis of this disease [34]. MicroRNA-505-3p is discussed as putative biomarker for primary biliary cirrhosis [35].

CDCA strongly affected genes involved in lipid homeostasis, including *FGF2*, *FGFR2*, *HMGCS2*, *FABP* and *APO* family members. As potent ligands for FXR, CDCA and other BAs regulate genes involved in

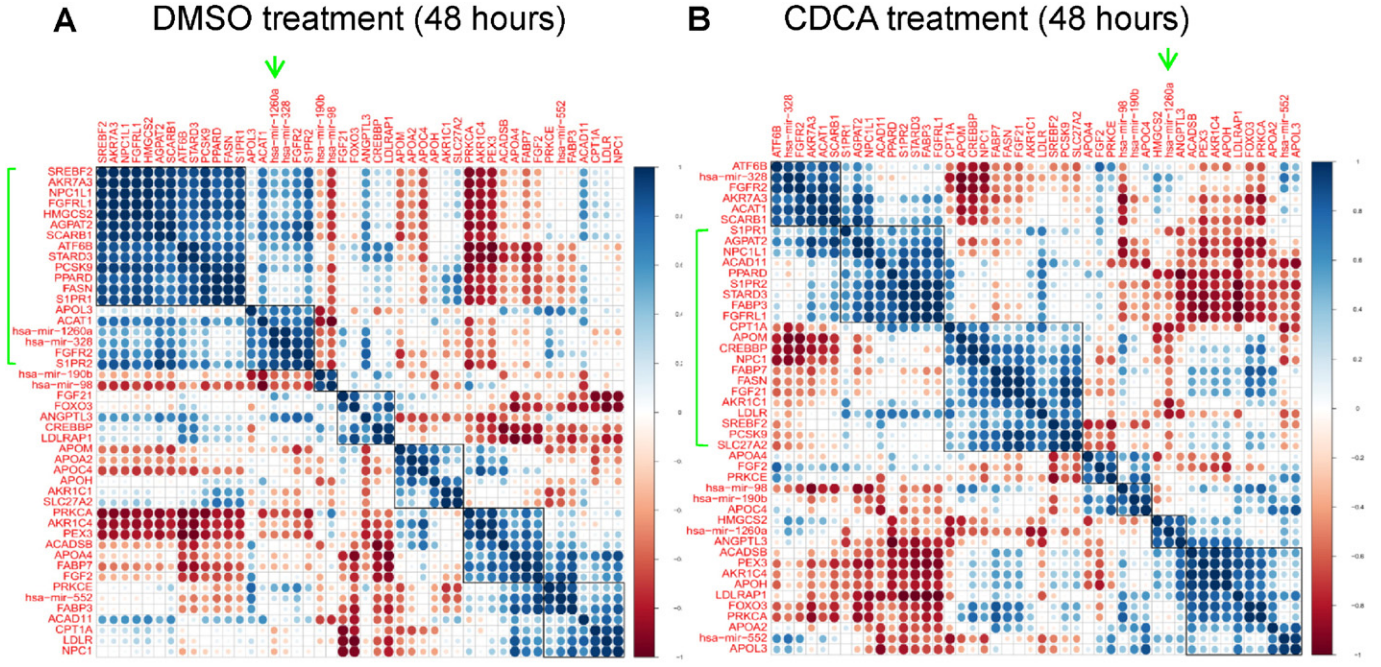


Fig. 5. Pearson's correlation analyses on mRNA and miRNA expression levels taking genes from the lipid gene cluster into consideration. DMSO treatment (A), CDCA treatment (B). Analyses included miRNAs significantly up- or downregulated and with predicted binding sites in at least three target genes within the lipid gene cluster. Shown are lipid cluster mRNA targets significantly up- or downregulated. Blue, positive correlation; red, inverse correlation. A distinct gene cluster composed of important lipid homeostasis regulating genes, such as S1PR2, CPT1A, APOM, AKR1C1 and LDLR is strongly inversely correlated with the expression of miR-1260a upon CDCA treatment (highlighted in green).

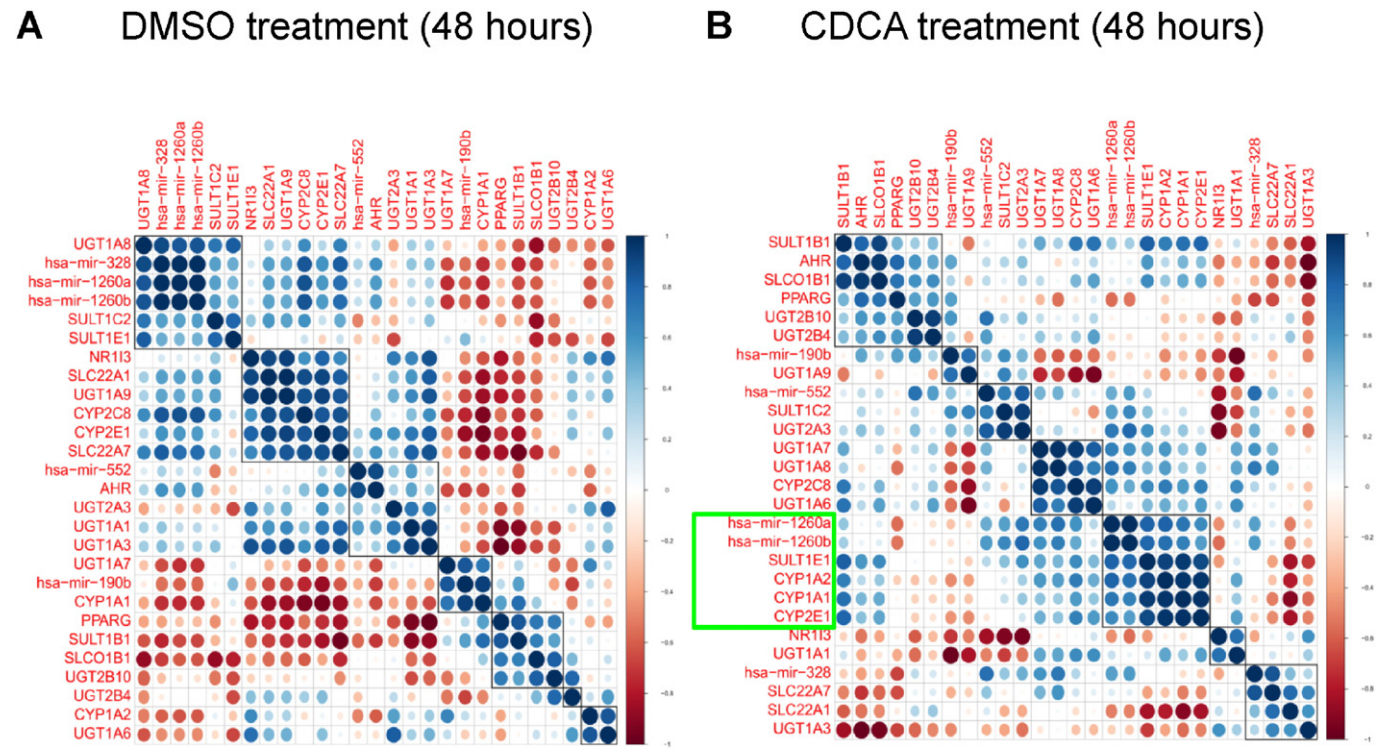


Fig. 6. Pearson's correlation analyses on mRNA and miRNA expression levels taking genes from the DM gene cluster into consideration. DMSO treatment (A), CDCA treatment (B). Analyses were performed including those miRNAs that were significantly up- or downregulated and for which binding sites in at least three target genes within the drug transport and metabolism (DM) gene cluster were predicted. Shown are DM mRNA targets that were significantly up- or downregulated. Blue, positive correlation; red, inverse correlation. A distinct gene battery, composed of important genes encoding drug metabolizing enzymes, including SULT1E1, CYP1A2, CYP1A1 and CYP2E1 and to a less extent several UGT family members and CYP2C8, is positively correlated with the expression of miR-1260a and miR-1260b upon CDCA treatment (highlighted in green).

cholesterol, FA, and lipid metabolism, either by direct transcriptional activation or by FGF-dependent hormonal signaling. In vivo studies performed in mice have shown that orally administered BAs lead to a positive effect on body weight [36] and lower the risk for NAFLD under high-fat diet conditions [2]. Our results support the notion that BAs modulate the expression pattern of genes involved in lipid synthesis, transport and metabolism, which could contribute to the lipid lowering properties repeatedly observed with BAs. In this context we would like to highlight the enhancement of miR-1260 expression upon CDCA treatment, which was associated with a strong parallel expression of genes involved in xenobiotica metabolism (CYP2E1, CYP1A1, CYP1A2) and an inverse expression of important genes involved in lipid homeostasis (i.e. CPT1A, APOM, LDLR). This finding points to a putative coregulatory element regulating the expression of miR-1260 and the mentioned drug gene group and a role of this microRNA as regulatory element in lipid metabolism. MicroRNA-1260 has been recently described as putative biomarker for paclitaxel-induced apoptosis in HCC cells [37]. Our systematic analysis of genes involved in drug metabolism and transport show, that many genes belonging to phase I (CYP1A members, CYP2E1, CYP2C8), phase II (several genes of the UGT and SULT family) and phase III (SLC22A1, SLC22A7) are significantly modulated by CDCA. This observation allows the speculation that bile acid derivates may have the potential to induce drug-drug interactions with other compounds through the modulation of DM gene expression. The importance of our findings with regard to a relevant interplay of BAs and DMEs is further underlined by the fact that distinct and relevant drug-drug interactions with BAs have been earlier described in other studies. This comprises amongst others the interaction between the immunosuppressant Ciclosporine A and bile acids. Ciclosporine A has been demonstrated to lead to a significant accumulation of bile acids and the development of a cholestatic feature in liver derived HepaRG cells [38]. Another important example comprises the interaction of the calcium channel blocker nitrendipine and CDCA. Sasaki and co-workers demonstrated that CDCA and UDCA are able to inhibit nitrendipine absorption by 50% and thus to decrease plasma concentrations of nitrendipine to a clinically relevant extent in healthy individuals [39].

Because the five batches of PHHs were obtained from patients resected for liver metastases that were incurred through different malignant tumors such as colon or kidney cancer, it cannot be excluded that the underlying pathologies or the existence of genetic polymorphisms within the discussed gene clusters or in genes coding for key transcription factors could induce an additional variance in gene expression. We specifically concentrated on the question to what extent CDCA is able to change the microRNA profile, as also optimally measurable in the chosen time frame of 48 h. It would be interesting to further study in future studies how and to what extent other epigenetic regulatory mechanisms, such as e.g. methylation or histone acetylation, are influenced by CDCA. Correlation analyses were done at the miRNA/mRNA level. Because the regulatory effect of miRNAs is often only observed at the protein level, we cannot exclude the possibility that the miRNAs have additional effects on targets that were not detected by assessment of mRNA levels alone.

We conclude that the expression of the human miRNAome and mRNAome in PHHs are significantly modulated by the bile acid CDCA, with relevant consequences for the functionality of gene networks involved in bile acid, lipid and drug metabolism. CDCA-induced regulation by miRNAs may exert downstream effects on genes within functionally important networks. Our findings give important novel insights into the ability of BA derivates to induce relevant changes in gene networks relevant for BA compound safety and metabolic disease development such as obesity and NALFD.

Conflicts of interest

None of the authors declares any conflicts.

Author contributions

RK, GK and JM contributed to study concept and design and data acquisition; RK, SML, WT and JM contributed to data acquisition; RK, AB, GK, and JM contributed to analysis of the data; RK, AB, GK, HBS and JM contributed to writing the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2016.04.037.

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